

CONCISE COMMUNICATION

Detection of Diverse Variants of Human Immunodeficiency Virus–1 Groups M, N, and O and Simian Immunodeficiency Viruses from Chimpanzees by Using Generic *pol* and *env* Primer Pairs

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Human immunodeficiency virus type 1 (HIV-1) infection of humans is the result of independent cross-species transmissions of simian immunodeficiency viruses (SIVcpz) from naturally infected chimpanzees (*Pan troglodytes troglodytes*) to man. To develop a polymerase chain reaction–based assay capable of detecting members of all major phylogenetic SIVcpz and HIV-1 lineages (groups M, N, and O), primer pairs in conserved *pol* and *env* regions were designed. Both primer sets amplified ≤ 10 copies of selected group M reference clones (subtypes A–H), proviral DNA or RNA of group N (YBF30), and group O of HIV-1 and also amplified divergent SIVcpz from cultured isolates (SIVcpzGAB1 and SIVcpzANT), uncultured spleen tissue (SIVcpzUS), and plasma (SIVcpzANT and SIVcpzUS). Sequences of the 2 amplicons (445 bp for gp41 and 261 bp for integrase) are of sufficient length for phylogenetic analyses, allowing both group and subtype classifications of the human viruses. Finally, both primer pairs are highly sensitive (>99%) in amplifying viral sequences from plasma taken from patients infected with HIV-1 group M ($n = 226$) and O ($n = 17$) viruses.

Globally circulating strains of human immunodeficiency virus type 1 (HIV-1) have been classified into 3 major phylogenetic groups, termed M, N, and O [1, 2], all of which cause AIDS in infected individuals. Group M (for “major”) comprises the great majority of HIV-1 infection worldwide and has been further subdivided into 12 distinct lineages, termed subtypes (A–D, F–H, and J), and circulating recombinant forms (AB, AE, AG, and AGI) [1]. Group O (for “outlier”) comprises many fewer isolates that are geographically restricted to west central Africa [3]. Finally, group N (for “non-M/non-O”) was discovered recently and is the least widespread of all HIV-1 lineages [2]. Viruses belonging to this group have thus far only been identified in a handful of individuals, all of whom were residents

of Cameroon [2]. Sequence variation among members of these various lineages is extensive, with nucleotide sequence variation in the *env* gene with a range of 18% among the different group M subtypes and circulating recombinant forms (CRFs) to nearly 42% among members of all 3 groups. Given this extent of diversity, it is not surprising that the great majority of nucleic acid–based diagnostic tests originally designed for group M, subtype B viruses have reduced sensitivities in detecting viruses from other groups and subtypes.

The need for screening tools that can detect the complete spectrum of HIV-1 variants has recently been reemphasized by the discovery of the likely primate reservoir of HIV-1. A recent study showed that members of 2 different subspecies of chimpanzees, the central *Pan troglodytes troglodytes* (*P.t.t.*) and the eastern *Pan troglodytes schweinfurthii* (*P.t.s.*), are infected with simian immunodeficiency viruses (SIVcpz) in the wild and that their respective viruses form 2 highly divergent, but subspecies-specific, phylogenetic lineages [4]. All HIV-1 strains known to infect humans, including members of the 3 groups of HIV-1, are closely related to viruses infecting *P.t.t.* Because hunting chimpanzees for food has become a commercialized trade, particularly in west central Africa [5], human exposure to SIVcpz has likely increased in the past 2 decades. It is possible that additional transmissions of this group of viruses have occurred

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but gone unrecognized because of the explosive spread of HIV-1 group M. Screening tools that can detect and distinguish divergent HIV-1 and SIVcpz strains in clinical specimens are urgently needed because studies of zoonotic disease emergence may gain greater public health attention.

The ideal screening tools would consist of a rapid serological test, combined with a molecular-based assay that can confirm the infection and classify the viruses with respect to existing or new HIV-1/SIVcpz phylogenetic lineages. Currently available serological tests are unable to distinguish the different SIVcpz/HIV-1 lineages. Thus, a polymerase chain reaction (PCR)-based approach that is cross-reactive, sensitive, and specific is needed. We report here 2 sets of *env* and *pol* primers capable of amplifying HIV-1 groups M, N, and O, as well as diverse SIVcpz. This highly sensitive and broadly cross-reactive PCR-based approach provides a new molecular tool to detect and distinguish genetically divergent variants of HIV-1 and SIVcpz in both human and chimpanzee populations.

Materials and Methods

Viral isolates and reference clones. Full-length molecular clones representing all major HIV-1 group M subtypes have been reported elsewhere [6]. Cultured HIV-1 isolates of groups M, O, and N (YBF-30) were generated by cocultivation procedure. The SIV isolates include 4 SIVsm (sooty mangaby), 3 SIVagm (African green), 1 SIVmnd (mandrill), 1 SIVsyk (sykes), and 1 SIVrcm (red cap mangaby). The SIV isolates from chimpanzees include SIVcpzGAB1 and SIVcpzANT.

Plasma specimens. A panel of reference plasma samples from known HIV-1-seropositive individuals, including specimens from persons in Uganda ($n = 53$), Cameroon ($n = 8$), Ivory Coast ($n = 34$), Ghana ($n = 16$), Republic of Congo ($n = 10$), Zimbabwe ($n = 8$), Mozambique ($n = 5$), Zaire ($n = 1$), South Africa ($n = 1$), Argentina ($n = 25$), Brazil ($n = 17$), United States ($n = 4$), Canada ($n = 1$), China ($n = 1$), Thailand ($n = 40$), Japan ($n = 3$), India ($n = 2$), Lebanon ($n = 7$), and Spain ($n = 7$) have been described elsewhere [7]. Plasma specimens were also obtained from 41 HIV-seronegative US blood donors and 16 HIV-2-positive west Africans. Plasma samples from 2 captive chimpanzees (Noah and Niko) infected with SIVcpzANT and uncultured spleen and plasma from another captive chimpanzee (Marilyn), who was infected with SIVcpzUS, were kindly provided by Drs. G. van der Groen and Larry Arthur, respectively [4].

PCR. Primers were designed on the basis of conserved integrase (intM-Z) and gp41 (gpM-Z) regions. The outer primers gp40F1 and gp41R1 of gpM-Z were described elsewhere [7]. The inner primers for nested PCR are gp46F2 (forward) 5'-ACA-ATTATTGTCTGGTATAGTGCAACAGCA-3' (nucleotides [nt] 7850-7879, based on HXB2, GenBank accession no. K03455) and gp48R2 (reverse) 5'-TCCTACTATCATTATGAATATTTT-TATATA-3' (nt 8265-8294). The outer primers for intM-Z are intF1 (forward) 5'-AGTGCTGCAGTTAAGGCAGCCTGTTG-GTGG-3' (nt 4596-4625) and intR1 (reverse) 5'-CTACCTGCC-ACACAATCATCACCTGCCATC-3' (nt 5051-5080); the inner primers are intF2 (forward) 5'-AAGAGATCAAGCTGAACA-

TCTTAAGACAGC-3' (nt 4724-4753) and intR2 (reverse) 5'-TGTATTACTACTGCCCTTCACCTTTCCA-3' (nt 4956-4984). Protocols for RNA extraction and conditions for reverse transcriptase-PCR and PCR analyses were as described elsewhere [7], with the exception of an annealing temperature of 55°C, which was used for the nested PCR with the intM-Z primer set.

Sequence and phylogenetic analyses. Selected nested-PCR products were directly sequenced by using dye terminator on an automated 377 DNA sequencer (Applied Biosystems, Foster City, CA). Sequences were aligned by using CLUSTALW [8], and phylogenetic trees were constructed by using the neighbor-joining method, which was implemented with the PHYLIP version 3.5c package [9].

Results

Amplification of HIV-1 group M, N, and O and SIVcpz viral sequences from reference clones and isolates. Both gpM-Z and intM-Z primer sets reliably amplified as few as 5-10 copies of full-length clones of group M subtypes A-H (figure 1A, 1B). Similarly, both primer pairs amplified viral RNAs from culture supernatants of group O isolates (BCF01, BCF02, BCF03, and 3012) and cultured peripheral blood mononuclear cell DNA from group N (YBF30) (figure 1C, 1D). Most important, both primers amplified SIVcpz derived from 2 chimpanzee subspecies: *P.t.t.* (SIVcpzGAB1 and SIVcpzUS) and *P.t.s.* (SIVcpzANT). However, none of the 4 SIVsm, 3 SIVagm, 1 SIVmnd, 1 SIVsyk, or 1 SIVrcm isolates were amplified with either primer set, although they all were amplified by SIV-specific integrase primers (data not shown). Thus, in contrast to our previously published gpM/O primer set, which does not amplify SIVcpz [7], the new primer sets gpM-Z and intM-Z can amplify members of all known HIV-1 and SIVcpz lineages.

Amplification of HIV-1 and SIVcpz viral RNA from plasma. We next examined the amplification efficiency of these primers in detecting plasma viremia from 226 group M (61 subtype A, 47 subtype B, 19 subtype C, 29 subtype D, 22 subtype E, 25 subtype F, 22 subtype G, and 1 subtype H) specimens and 17 group O specimens (figure 1E, 1F). Of the 226 group M specimens, 224 (99%) were amplified by gpM-Z primers and 225 (99.5%) by intM-Z primers. One subtype B specimen from Brazil was missed by both primers, whereas 1 subtype G from the Ivory Coast was missed only by gpM-Z. In addition, all 17 group O specimens were amplified by both primers. No plasma specimens from persons infected with group N viruses were available. Finally, both primer sets yielded positive amplifications from chimpanzee plasmas (SIVcpzUS and SIVcpzANT). By contrast, plasmas from HIV-2-infected patients ($n = 16$) and HIV-1-negative blood donors ($n = 41$) gave no amplifications (data not shown).

Phylogenetic classification. Phylogenetic analysis of primate lentiviral sequences provides the basis for their classification into groups and subtypes for HIV-1 and into major lineages for SIVcpz. We thus examined whether sequences de-

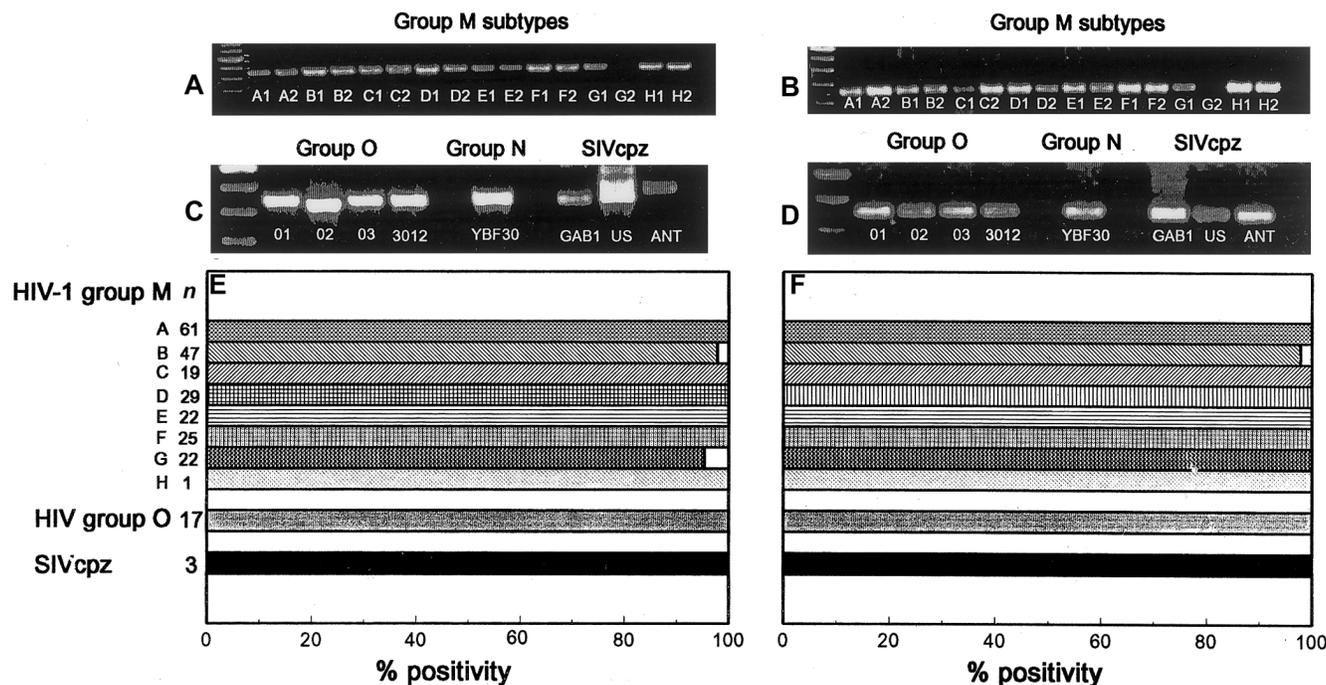


Figure 1. Detection of diverse strains of human immunodeficiency virus type 1 (HIV-1) groups M, N, and O and simian immunodeficiency viruses (SIVcpz) by using gpM-Z (panels A, C, E) and intM-Z (panels B, D, F) primer sets. A and B, Known copy numbers (10 and 5 copies per polymerase chain reaction, abbreviated as A1, A2, etc., for each subtype) of reference clones of HIV-1 group M subtype A (92UG037.1), B (YU2), C (92BR025.8), D (94UG114.1), AE (90CF402.8), F (93BR020.1), AG (92NG003.1), and H (90CF056.1) were amplified by using gpM-Z (A) and intM-Z (B). C and D, Amplification of viral RNA (group O [BCF01, 02, 03, and 3012]) and DNA (group N [YBF30]) and SIVcpz (GAB1, US, and ANT) by primer pairs gpM-Z and intM-Z. E and F, Detection of plasma viral RNA from patients infected with HIV-1 group M (subtypes A-H; n, no. of specimens) and group O and from chimpanzees infected with SIVcpzUS and SIVcpzANT (Niko and Noah) by primers gpM-Z and intM-Z. The y axis represents the percentage of specimens that were positive.

rived from the amplicons were of sufficient length to provide reliable phylogenetic information. Selected PCR products from 143 group M (A = 22, B = 34, C = 19, D = 24, E = 22, F = 9, G = 12, and H = 1), group O (n = 17), group N (YBF30), and SIVcpz (US, GAB1, ANTnoah, and ANTniko) specimens were sequenced and used for phylogenetic tree construction. Figure 2A depicts the relationships of sequences derived from the amplified integrase region. The topology shows overall branching orders very similar to the orders reported for full-length *pol* sequences [4]. Newly amplified group M and O viruses (shown in bold) are readily identified as such because they cluster with previously characterized reference sequences. YBF30, the single representative of HIV-1 group N, clusters as expected, between groups M and O. Finally, the relative positions of the PCR-derived SIVcpzUS, SIVcpzGAB1, and SIVcpzANT are consistent with those identified elsewhere for their full-length sequences [4]. The only shortcoming of the amplified integrase region is that it is not suitable (because of its length) for more detailed subtype classification of group M viruses. However, such classification is possible for sequences derived from the gp41 amplicons. As reported elsewhere [10] and shown in figure 2B, a phylogenetic tree constructed from the gp41 sequences

yields an overall topology that is very similar to that of a full-length *env* tree [6,10]. Thus, 28 newly characterized group M viruses (depicted in bold) are readily classified into group M subtypes A (4), B (4), C (4), D (5), E (3), F (3), G (4), and H (1). Finally, gp41 sequences can also be used to classify new SIVcpz strains. Sequence amplified from the plasma of a chimpanzee (Niko) experimentally infected with SIVcpzANT cluster very closely with sequence derived from the plasma of the chimpanzee (Noah) naturally infected with this isolate. Taken together, a PCR-based approach that uses both intM-Z and gp41M-Z primers should be able to type newly identified HIV-1 and SIVcpz strains.

Discussion

In this study, we designed primers on the basis of conserved regions of the HIV-1 and SIVcpz genomes, termed as intM-Z and gpM-Z, and confirmed their sensitivity and specificity with a panel of reference strains. The results show that the 2 primer sets not only detect HIV-1 group M (subtypes A-H; including F2 and J [data not shown]), group N, and group O viruses but also detect diverse SIVcpz viruses, including the highly diver-

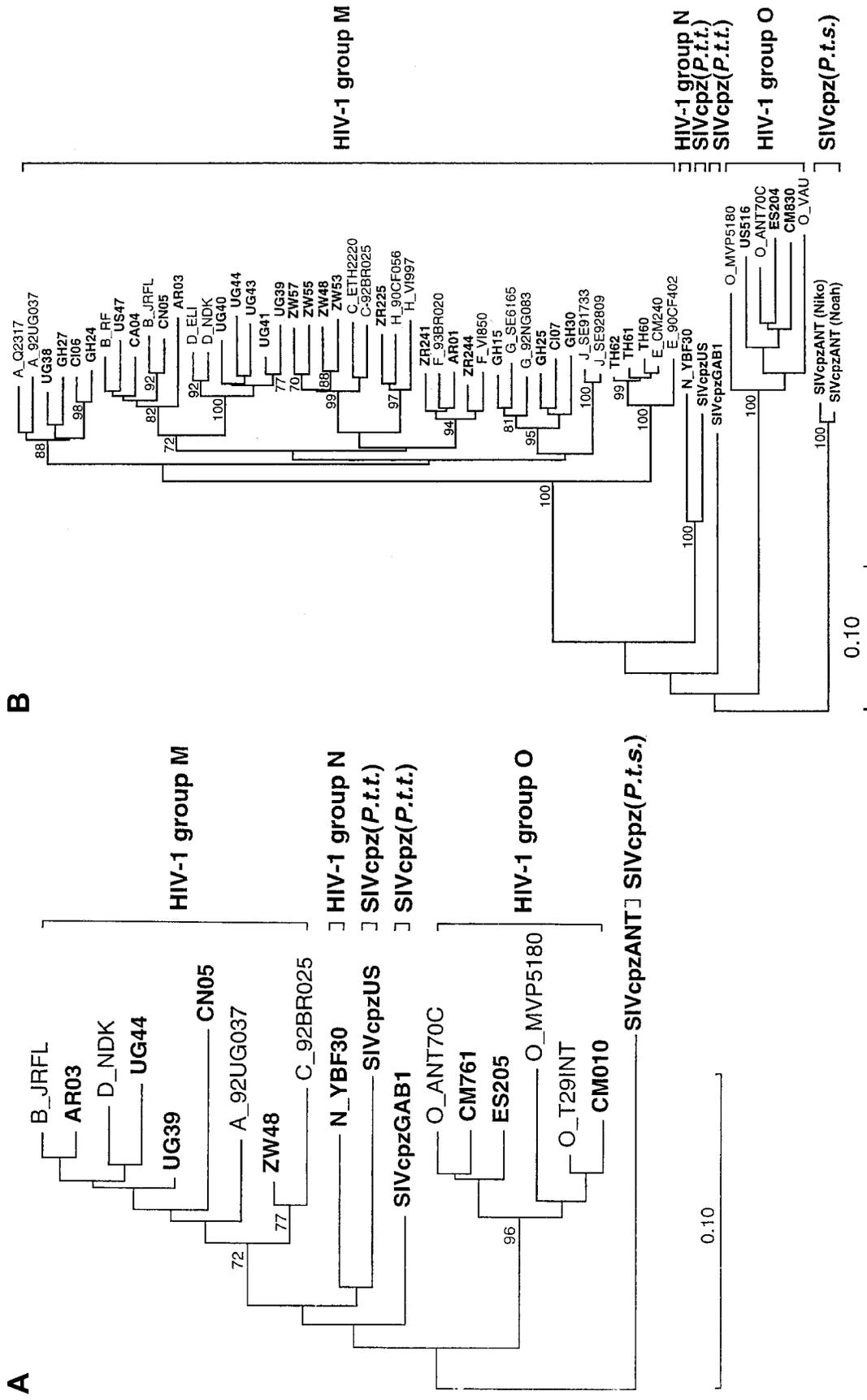


Figure 2. Phylogenetic position of newly sequenced (*bold/ace*) and previously reported human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIVcpz) strains in subgenomic *pol* (IN) (A) and *env* (gp41) (B) regions. A, Relationships of sequences derived from the amplified IN region; B, gp41 Region. The topology shows an overall branching order consistent with phylogenies reported elsewhere for full-length *pol* [4] and *env* [6, 10] sequences. Trees were derived from nucleotide-sequence alignments (consensus lengths of 183 and 348 bp, respectively) by using the neighbor-joining method. Horizontal branch lengths are drawn to scale, with the bar indicating 0.10 nucleotide substitutions per site. Numbers at the nodes indicate the percentage of bootstrap values (of 500) in which the cluster to the right is supported (only values $\geq 70\%$ are shown). Brackets identify known HIV-1 (groups M, N, and O) and SIVcpz (*Pan troglodytes troglodytes* and *Pan troglodytes schweinfurthii*) viral lineages.

gent SIVcpzANT strain. Although molecular diagnostic techniques have been reported for DNA and/or RNA detection of HIV-1 infections [7, 9, 11], this is the first molecular approach that reliably detects representatives of all major lineages within the HIV-1/SIVcpz radiation. Thus, the PCR-based approach reported here provides a new and unique tool for studying the molecular epidemiology and transmission patterns of both groups M and non-M HIV-1 infections and potential variants that may have not yet been recognized.

Recent data indicate that human HIV-1 infection represents a zoonosis and that there may have been as many as 3 independent transmissions from chimpanzees to humans [4]. The exact circumstances of these transmissions remain unknown, but it is likely that transmission occurred when humans were exposed to blood or other body fluids from infected chimpanzees in the course of hunting or preparing meat for consumption, were bitten or scratched by infected pets or wounded animals, or consumed raw meat containing infectious viruses. Commercial logging of tropical forests is an important economic activity for several African countries, and this practice has resulted in new road construction, human migration, and development of social and economic networks supporting this industry [5]. These changes have enabled hunters to penetrate previously inaccessible forest areas and make use of newly developed infrastructure to capture and transport forest wildlife, or "bushmeat," from remote areas to major city markets [5]. Thus, the potential for human exposure to primate lentiviruses, including SIVcpz, has likely increased, along with the conditions needed to support the subsequent emergence of new zoonotic infections [12].

Although it seems unlikely that another zoonotic transfer could cause an epidemic of the magnitude of the current HIV-1 pandemic, the possibility of ongoing SIVcpz transmissions to humans, and of recombination between currently circulating and newly introduced viral strains resulting in changed biologic properties [13, 14], merits public health attention. To assess additional zoonotic threats, researchers need data on the prevalence of SIVcpz infections in wild living adult chimpanzees throughout the primate's natural ranges. Chimpanzees are highly endangered, and the development of noninvasive diagnostics capable of detecting SIVcpz infection in urine or fecal material is essential. Amplification of viral RNA from fecal material of HIV-1-infected humans has been reported [15], and a similar approach is thus likely to work for naturally infected chimpanzees. Surveys are also needed for humans with a high likelihood of exposure to SIVcpz, including individuals involved in hunting or capturing of these animals, preparing their meat, or other related purposes. The primer pairs reported here should facilitate such approaches, because they are capable of amplifying highly divergent SIVcpz strains from a variety of different tissues and starting materials.

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